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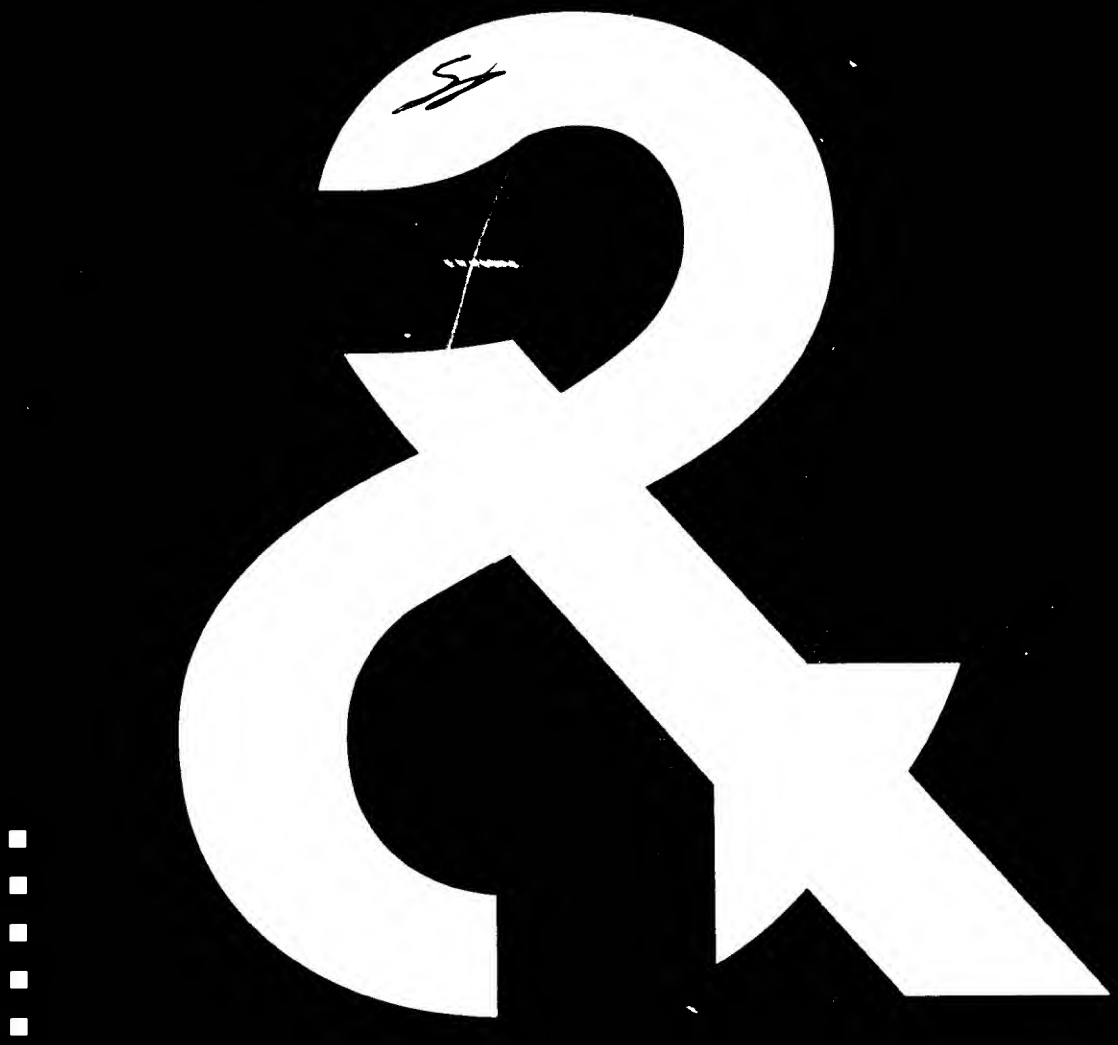

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Association of Dexamethasone-induced Apoptosis and G_1-Arrest of Human Leukemic CEM Cells with Polyamine Deficit

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The effects of DFMO or/and putrescine on the dexamethasone-induced apoptosis of CEM cells were studied to investigate the role of polyamines in anti-leukemic glucocorticoid action. Dexamethasone-induced apoptosis was preceded by significant decreases of cellular polyamine contents and putrescine uptake activity. But DFMO produced decreases of putrescine and spermidine contents and marked increase of putrescine uptake activity, but did not induce apoptosis. However, dexamethasone and DFMO, respectively, induced G_1-arrest in cell cycle and hypophosphorylation of pRb, resulting in the increase of G_1 to S ratio and decrease of CEM cell count. DFMO enhanced the dexamethasone-induced apoptosis and G_1-arrest. On the other hand, putrescine little affected the apoptotic and G_1-arresting activities of dexamethasone, but almost suppress the effects of DFMO and also the DFMO-dependent enhancement of dexamethasone effects. These results suggested that the dexamethasone-induced apoptosis to be associated with pRb hypophosphorylation and G_1-arrest in CEM cells might be ascribed to the concomitant decreases of cellular polyamine contents and putrescine uptake activity.

Key Words: Leukemic cell, Dexamethasone, DFMO, Polyamine, Putrescine uptake, Apoptosis, pRb

INTRODUCTION

Glucocorticoids have therapeutic potentials of anti-inflammatory and immunosuppressive effects, and induce apoptosis of lymphocytes (Diasio & LoBuglio, 1996). And the lympholytic activity of glucocorticoids has been proved to be related to their apoptotic activity on lymphocytes (Wyllie, 1980). Dexamethasone has been well known to lead prompt remission in acute lymphoblastic leukemia (Chabner et al, 1996).

Polyamines are essential for cell growth, proliferation, and differentiation (Tabor & Tabor, 1984; Pegg, 1986; Heby & Persson, 1990), and so have some roles in apoptosis (Brooks, 1995). Polyamines, particularly spermine, have been reported to show the inhibitory effect on the apoptosis induced by dexamethasone (LaVoie & Witorsch, 1995), Ca^{2+} ionophore (Brune et al, 1991), and topoisomerase inhibitors (Solary et al, 1996; 1994; Bertrand et al, 1993) in thymocytes and lymphocytes of leukemia and lymphoma.

On the contrary, several reports demonstrated that excessive polyamine accumulation might induce apoptotic cell death (Pegg et al, 1995; Poulin et al, 1995), and that a stable polyamine analogue also induced both growth inhibition and apoptosis of cancer cells (McCloskey et al, 1995). And in the mouse myeloma cells to overexpress ornithine decarboxylase (ODC), the addition of ornithine to the culture medium increased cellulr putrescine content, resulting in apoptotic cell death (Tobias & Kahana, 1995).

In the early phase of the rat thymocyte apoptosis induced by glucocorticoid, heat shock, and γ-irradiation, an increased expression of ODC gene appears with the decrease of cellular polyamine contents,

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before laddering of genomic DNA (Desiderio et al, 1995; Grassilli et al, 1995).

The cellular levels of polyamines are regulated by the activities of polyamine transporters (Pegg, 1988; Lessard et al, 1995) as well as by the activities of synthetic enzymes, ODC and S-adenosylmethionine decarboxylase (SAM-DC), spermidine/spermine N^1-acetyltransferase, and oxidases (Pegg, 1988; Seiler, 1987; Casero & Pegg, 1993).

This study was carried out to investigate the role of polyamines in the dexamethasone-induced apoptotic cell death of CCRF-CEM cells, a human T lymphoblastic leukemia cell line (Foley et al, 1965) which has been considered to be labile to dexamethasone cytotoxicity (Norman & Thompson, 1977; Yuh & Thompson, 1989), referring the effect of the polyamine depletion by DL-α-difluoromethylornithine (DFMO) (Mamont et al, 1978).

METHODS

Cell culture and drug treatment

CEM cells (ATCC CCL 119) were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (Gibco) under humidified atmosphere of 5% CO_2 at 37°C. Two hours after beginning of cell culture (seeding density of $2 \pm 0.2 \times 10^5$ cells/ml), DFMO (1×10^{-3} M; control, PBS 0.1% v/v of medium; Marion Merrell Dow) or dexamethasone (1×10^{-6} M; control, ethanol 0.1%) was added to the culture medium, and the polyamine contents and putrescine uptake activity of CEM cells were measured. And 2 hours, 14 hours, and 16 hours after beginning of cell culture, DFMO, putrescine (1×10^{-5} M; control, PBS 0.1%), and dexamethasone were respectively added to the culture medium, and then the studies of MTT assay, LDH release, DNA fragmentation, cell cycle analysis, and phosphorylation of Rb protein were performed with 24 hour intervals or at 72 hour after dexamethasone treatment.

Measurement of cellular polyamine contents

The CEM cells of ($2 \pm 0.2 \times 10^6$) were washed twice with PBS. The washed pellet was extracted with 200 μl of 0.6 M perchloric acid at 4°C for 30 min and then centrifuged at 12000 × g for 5 min. The supernatant of 100 μl was evaporated to dryness. For derivatization of polyamines, the dry residue dissolved in 100 μl of 1 M sodium carbonate was mixed with 300 μl of FNBT reagent (1% 4-fluoro-3-nitrobenzotrifluoride solution in DMSO; Aldrich) and incubated at 60°C for 20 min. At the end of the reaction, 40 μl of 1 M histidine in 1 M sodium carbonate was added to the solution, and the reaction was continued for further 5 min. After cooling the reaction mixture, the polyamine derivatives extracted twice with 2 ml of 2-methylbutane (Aldrich) were dried and dissolved in methanol. The methanol solution was applied on an ODS reverse phase column of HPLC, which was eluted by an isocratic mobile phase of 80% acetonitrile, monitoring at 242 nm (Spragg & Hutchings, 1983).

[^{14}C]Putrescine uptake measurement

The CEM cells ($1 \pm 0.1 \times 10^6$/ml) were resuspended in PBS and then incubated with 1 μM ~ 20 μM of [^{14}C]putrescine (Amersham) at 37°C for 60 min under 5% CO_2 atmosphere. At the end of incubation, cold PBS of 1 ml containing 1 mM putrescine was added to the cell suspension and centrifuged at 2000 × g for 5 min. The pellet was washed twice with PBS and dissolved in 200 μl of 1 N NaOH at 60°C for 60 min. The solution was neutralized with 200 μl of 1 N HCl, and the radioactivity was measured by β-scintillation counter (Porter et al, 1985; Lessard et al, 1995). The uptake parameters were determined by Edie-Scatchard plot.

MTT assay

The culture medium (100 μl) containing suspended CEM cells was incubated with 10 μl of MTT labeling reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 5 mg/ml in PBS; Boehringer Mannheim), at 37°C for 4 hours. And the formazan crystal produced by cellular dehydrogenases was resolved in 100 μl of solubilizing solution (10% SDS in 0.01 M HCl; Boehringer Mannheim) at 37°C for 12 hours, and then the absorbance was measured at 600 nm (Mosmann, 1983).

LDH release assay

The supernatant (50 μl) of culture medium was reacted with 50 μl of LDH substrate solution (Promega)

for 30 min, and the reaction was stopped by addition of 50 μl of 1 M acetic acid. The activity of LDH released was measured as the absorbance at 492 nm (Korzeniewski & Callewaert, 1983; Decker & Lohmann-Matthes, 1988).

DNA fragmentation assay

The CEM cells ($2 \pm 0.2 \times 10^6$) washed with PBS were digested in 100 μl of lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0, and 0.8% sodium lauryl sarcosine) and 50 μl of RNase A (1 mg/ml) at 37°C for 90 min, and then incubated overnight with 50 μl of proteinase K (20 mg/ml) at 50°C. The genomic DNA was extracted once with phenol- chloroform-isoamylalcohol (25:24:1) and twice with chloroform-isoamylalcohol (24:1). DNA was precipitated by adding ammonium acetate upto 2.5 M and absolute ethanol. Being incubated for more than 2 hours at 4°C, the DNA was pelleted by centrifugation 12000 $\times$g for 15 min and washed with 70% ethanol. The DNA pellet was dried and dissolved in TE buffer (pH 8.0), and the fragmented DNA of 10 μg was separated on a 2% agarose gel, which was stained with ethidium bromide and visualized under UV light.

Flow cytometric analysis of cell cycle

The PBS-washed CEM cells ($1 \pm 0.1 \times 10^5$) were fixed in 2 ml of cold 70% ethanol at 4°C for 60 min, and then washed with PBS and resuspended in 0.5 ml of PBS. To this cell suspension, 0.5 ml of RNase (1 mg/ml in PBS) and 1 ml of propidium iodide (100 μg/ml in PBS) were added. And the cellular distributions to G_1, S, and G_2/M phases were analyzed by a flow cytometer (Nicoletti et al, 1991).

Western blot analysis of phosphorylated Rb protein

The CEM cells ($6 \pm 0.2 \times 10^6$) washed with PBS were lysed in SDS-PAGE sample buffer containing dithiotreitol, and boiled for 5 min. The protein content of PBS-washed cells was measured by Bradford's method (1976). Each lysate was electrophoresed on a 7.5% SDS-PAGE gel and transferred into PVDF membrane. Rb immunoblot was probed using the anti-human Rb monoclonal Ab (Pharmingen), and detected with ECL-blotting system containing HRP-labelled secondary Ab of mouse Ig (Amersham).

RESULTS

Effects of DFMO and dexamethasone on the polyamine metabolism in CEM cells

The change of polyamine contents was measured after DFMO and dexamethasone treatment to evaluate the time-dependent change of polyamine metabolism by DFMO or dexamethasone in CEM cells (Fig. 1). Putrescine content was almost depleted to less than 2% of control after 8 hours of DFMO treatment, and by dexamethasone, it was significantly decreased to about 50% of control at 8 hour and nearly depleted to that by DFMO after 24 hours. Spermidine content was almost depleted after 24 hours of DFMO

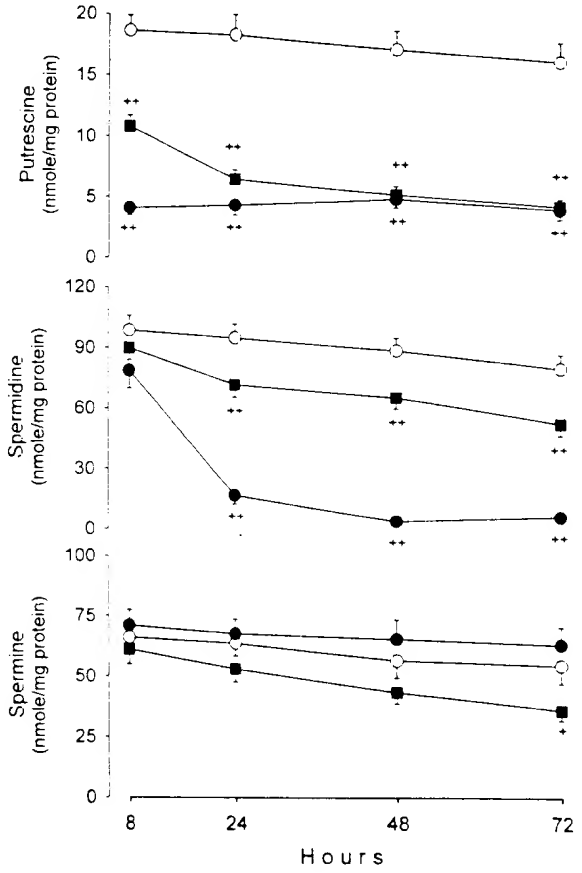

Fig. 1. Effects of DFMO (10^{-3} M) and dexamethasone (DX: 10^{-6} M) on the polyamine contents of CEM cells.
○: Control, ●: DFMO, ■: DX
Each point represents mean + S.E. of 4 independent measurements.
+ and ++ indicate $p < 0.1$ and $p < 0.05$, in comparison to the control group.

Table 1. Effects of DFMO and dexamethasone on the [^{14}C]putrescine uptake of CEM cells

Treatment	Km (uM)	Vmax (nM/hr/mg protein)
Control	22.42 ± 2.30	2.14 ± 0.19
DFMO 12 hr	8.31 ± 1.21**	7.69 ± 0.81**
DFMO 24 hr	6.85 ± 0.89**	15.24 ± 1.17**
DFMO 48 hr	6.96 ± 0.75**	27.93 ± 2.13**
DFMO 72 hr	7.05 ± 0.79**	1.53 ± 1.96**
DX 24 hr	17.16 ± 1.94	1.12 ± 0.21**
DX 48 hr	18.31 ± 1.95	1.20 ± 0.29**
DX 72 hr	17.20 ± 1.87	1.14 ± 0.27**
DFMO 36 hr & DX 24 hr	9.48 ± 0.86	16.98 ± 1.71**
DFMO 60 hr & DX 48 hr	2.15 ± 1.02**	9.71 ± 0.89**
DFMO 84 hr & DX 72 hr	12.61 ± 0.93**	9.29 ± 1.32**

** indicates $p < 0.05$

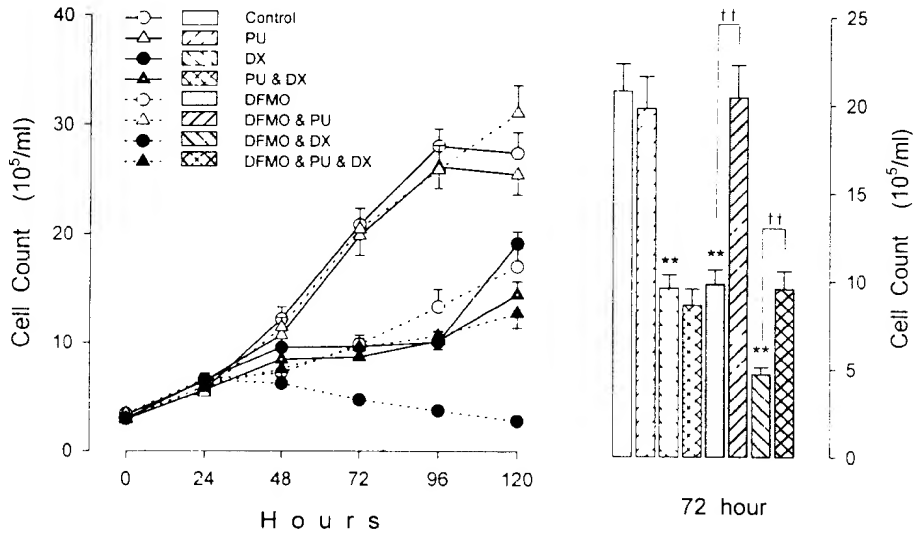

Fig. 2. Effects of DFMO and putrescine (PU, 10^{-5} M) on the change of CEM cell count by dexamethasone.
** indicates $p < 0.05$, in comparison to the control count at 72 hour.

treatment, and by dexamethasone, it was decreased to less than 80% of control after 24 hours. Finally, spermine content was not affected by DFMO, and by dexamethasone, the decrease of spermine content was continued till 72 hour after treatmemt.

CEM cells showed Km and Vmax of putrescine uptake as follows; 22.4 ± 2.3 μM and 2.14 ± 0.19 nM/hour/mg protein, respectively (Table 1). DFMO significantly decreased the Km at 12, 24, 48, and 72 hour, and significantly increased the Vmax at 12 hour and further increased that after 24 hours. But dexamethasone slightly decreased the Km at 24, 48, and 72 hour, and significantly decreased the Vmax. DFMO, treated at 12 hour prior to dexamethasone, enhanced the slight decrease of Km by dexamethasone, and reversed the decrease of Vmax by dexamethasone.

Effects of DFMO and putrescine on the inhibition of cellular proliferation by dexamethasone

Cell counting and MTT assay were performed to evaluate the effects of DFMO and putrescine on the change of cellular proliferation by dexamethasone (Fig. 2 & 3).

The cell count and the MTT value of CEM cells increased for 4 days, and this increase was significantly inhibited by dexamethasone. DFMO also significantly inhibited the increase of cell count and MTT value, and enhanced the inhibitory effects of dexamethasone. Otherwise, putrescine had little effect on the increase of cell count and MTT value of CEM cells, and little affected the inhibitory effect of dexamethasone on the cell count and MTT value. But putrescine almost reversed the inhibitory effect on the cell count and MTT value by DFMO, and also reversed the enhancement of dexamethasone effects by DFMO. The MTT value of culture well may represent the count of viable cells in the well, and the absorbance per viable cell, which declined slowly, was affected by neither dexamethasone nor DFMO (Fig. 3-lower).

Effects of DFMO and putrescine on the apoptotic cell death by dexamethasone

LDH release and DNA fragmentation were measured to examine the effects of DFMO and putrescine on the apoptotic cell death induced by dexamethasone (Fig. 4 & 5).

The LDH release from CEM cells and the fragmentation of DNA appeared not to be significant for 72 hours, but dexamethasone significantly increased LDH release after 48 hours and induced evident fragmentation of DNA at 72 hour. DFMO had little effect on LDH release and induced little fragmentation of DNA. But DFMO significantly enhanced the increase of LDH release by dexamethasone (Fig. 4-lower), and also enhanced the DNA fragmentation by dexamethasone. Putrescine had little effect on the LDH release and DNA fragmentation of CEM cells, and little affected the increase of LDH release and

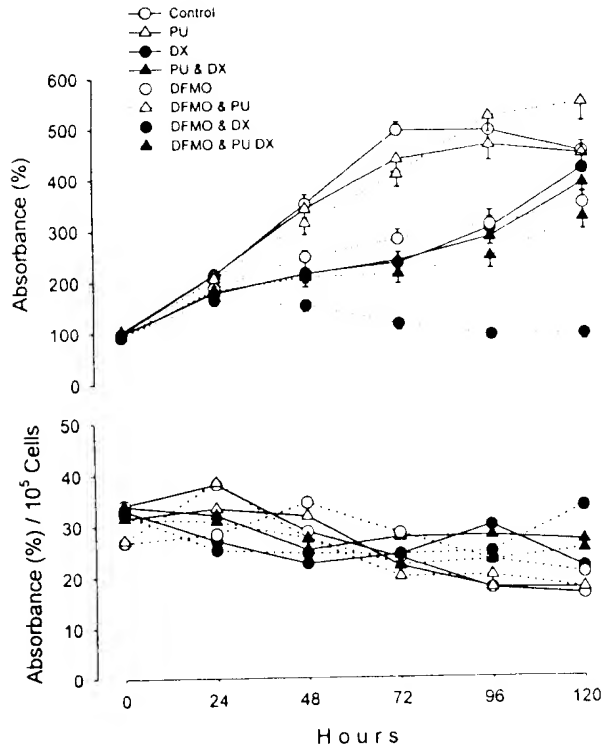

Fig. 3. Effects of DFMO and putrescine on the change of MTT value of CEM cells by dexamethasone.

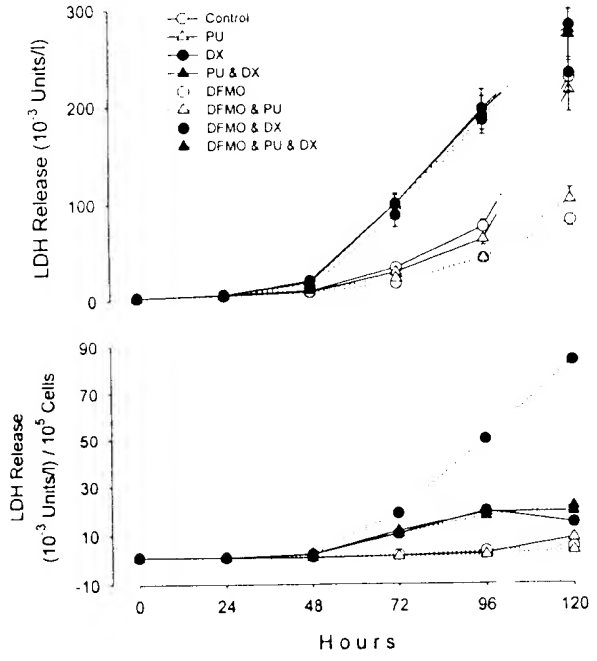

Fig. 4. Effects of DFMO and putrescine on the increase of LDH release from CEM cells by dexamethasone.

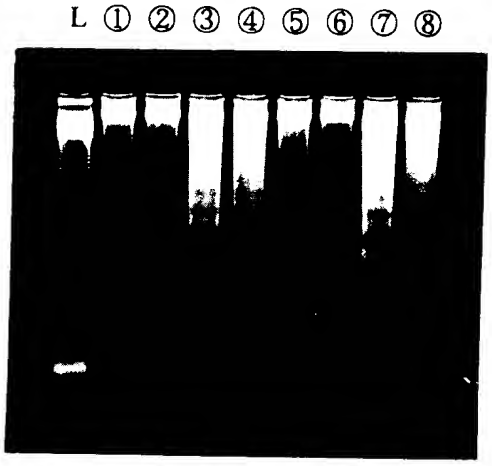

Fig. 5. Effects of DFMO and putrescine on the change of DNA fragmentation at 72 hour after dexamethasone treatment.
lane L: Authentic marker of 123 bp DNA ladder,
lane 1: Control, lane 2: PU,
lane 3: DX, lane 4: PU & DX,
lane 5: DFMO, lane 6: DFMO & PU,
lane 7: DFMO & DX, lane 8: DFMO & PU & DX

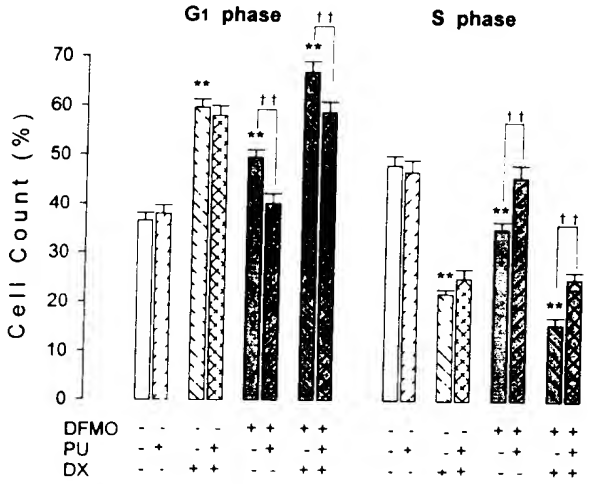

Fig. 6. Effects of DFMO and putrescine on the change of cell cycle distribution of CEM cells by dexamethasone.

Table 2. Effects of dexamethasone, DFMO, and putrescine on the ratio of G_1 to S phase distribution of CEM cells

Treatment	(−) Putrescine	(+) Putrescine
Control	0.77 ± 0.06	0.83 ± 0.08
DX	2.75 ± 0.20**	2.37 ± 0.25
DFMO	1.42 ± 0.11**	0.89 ± 0.09 ‡‡
DFMO & DX	4.41 ± 0.56**	2.39 ± 0.23 ‡‡

** indicates $p < 0.05$ in comparison to the control, and ‡‡ indicates $p < 0.05$ in comparison to the (−) putrescine.

DNA fragmentation by dexamethasone. But putrescine almost reversed the enhancement of dexamethasone-induced LDH release (Fig. 4-lower) and DNA fragmentation by DFMO.

Effects of DFMO and putrescine on change of cell cycle by dexamethasone

Cell cycle profile was analyzed to assess the effects of DFMO and putrescine on the cell cycle arrest by dexamethasone and to elucidate the relation between the cell cycle arrest and subsequent development of apoptosis.

Cell cycle distributions of CEM cells were as follows; 36.6 ± 1.5% in G_1, 48.1 ± 1.9% in S, and 15.4 ± 1.2% in G_2/M phase at 72 hour, and dexamethasone significantly increased the cellular distribution in G_1 phase and reciprocally decreased the distribution in S phase, with persistent percentage in G_2/M phase. DFMO also increased the ratio of cellular distribution in G_1 phase to the distribution in S phase, with no change in G_2/M phase, and DFMO enhanced the G_1-arrest by dexamethasone. Putrescine little affected the cell cycle progression as well as the G_1-arrest by dexamethasone, but it almost reversed the G_1-arrest by DFMO and also reversed the enhancement of dexamethasone-induced G_1-arrest by DFMO (Fig. 6 & Table 2).

Effects of DFMO and putrescine on the change of pRb phosphorylation by dexamethasone

The phosphorylation of pRb has been known to closely relate to the cell cycle progression through G_1 phase, and we analyzed the phosphorylation status of pRb to explain aspects of the effects of DFMO and putrescine on G_1-arrest induced by dexamethasone.

The pRb of CEM cells was highly phosphorylated

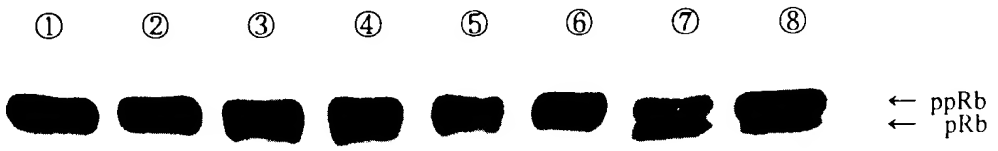

Fig. 7. Effects of DFMO and putrescine on the change of Rb protein phosphorylation at 72 hour after dexamethasone treatment.
lane 1: Control, lane 2: PU,
lane 3: DX, lane 4: PU & DX,
lane 5: DFMO, lane 6: DFMO & PU,
lane 7: DFMO & DX, lane 8: DFMO & PU & DX

with normal cell cycle progression, and dexamethasone hypophosphorylated pRb at 72 hour of treatment (Fig. 7). DFMO also inhibited the phosphorylation, and enhanced the hypophosphorylation of pRb by dexamethasone to display an evident band of hypophosphorylated. Putrescine had little effect on the normal hyperphosphorylation and dexamethasone-induced hypophosphorylation, but putrescine reversed the hypophosphorylation of pRb by DFMO and also reversed the enhancement of dexamethasone-induced hypophosphorylation by DFMO.

DISCUSSION

The ODC and polyamines have been known to involve in dexamethason-induced apoptosis of thymocytes (Desiderio et al, 1995; Grassilli et al, 1995), and the apoptosis was significantly inhibited by addition of polyamines to the culture medium (Brune et al, 1991).

In this study, DFMO almost depleted cellular putrescine and spermidine but little affected spermine content of CEM cells. Dexamethasone produced acute depletion of cellular putrescine, followed by gradual decrease of spermidine and spermine contents. DFMO enhanced the putrescine uptake activity within 12 hours of treatment, but dexamethasone depressed the Vmax of putrescine uptake althouth the putrescine content was almost depleted. Though the polyamine transporters have been known to be upregulated by the decrease of cellular polyamine contents (Grillo et al, 1989), little resource of polyamines in culture medium made the transporters to be useless and then the putrescine and spermidine contents remained to be depleted in DFMO-treated CEM cells. However, the spermine content was little changed by DFMO in this study, and this result seemed to be related to previous studies which showed the different responsivenesses of the cellular spermine contents to DFMO in different cell lines (Pera et al, 1986; Dorhout et al, 1995; Walters & Wojcik, 1994).

The dexamethasone-induced acute depletion of cellular putrescine and the following decrease of higher polyamine contents in this study might be associated with the reduction of ODC activity in CEM cells. Also, ODC expression was inhibited in the TGF β_1-induced apoptosis of L1210 cells (Grzelkowska et al, 1995). But several reports opposed above suggestion; the early expression and increase in activity of ODC associated with the decrease of polyamine contents were preceded to the appearance of DNA laddering in dexamethasone-induced apoptosis of thymocytes (Desiderio et al, 1995; Grassilli et al, 1995), and the c-Myc-induced apoptosis was associated with the expression and activation of ODC (Packham & Cleveland, 1994; 1995; Packham et al, 1996). Nevertheless, there are several evidences that the decrease of cellular ploy amine contents is one of the characteristic features of dexamethasone-induced apoptosis (Desiderio et al, 1995; Grassilli et al, 1995). There may be putative contributors to decrease polyamine contents in the process of apoptosis, besides the changes of activities of the synthetic enzymes (Desiderio et al, 1995); reduced cellular availibility of ornithine and arginine as substrates, oxidative metabolism by diamine and polyamine oxidases (Buttke & Sandstrom, 1994), increased acetylation facilitating the excretion of polyamines (Casero & Pegg, 1993), activation of transglutaminase (Piacentini et al, 1991; Martin et al,

1994) which can use polyamines as substrates (Fesus et al, 1991).

The increase of putrescine uptake activity by DFMO was in accordance with the other studies using leukemia and other cell lines (Kramer et al, 1993; Byers & Pegg, 1989; Walters & Wojcik, 1994), and the decrease of putrescine uptake activity can be considered to be one feature in dexamethasone-induced apoptotic cell death. However it is remained to be clarified wheather the decreases of polyamine contents and putrescine uptake activity are primary or secondary to the apoptosis induced by dexamethasone.

CEM cells continued to proliferate for 4 days without refresh of culture medium and showed slight decline of cellular activity in MTT assay after 24 hours. Both of DFMO and dexamethasone inhibited the proliferation of CEM cells, and dexamethasone increased LDH release from the cells and fragmentation of DNA, which were enhanced by DFMO. In addition, exogenous putrescine reversed the enhancement of dexamethasone-induced actions by DFMO as well as the antiproliferative action of DFMO. These results seemed to be in accordance with the previous studies, in which exogenous putrescine reversed the DFMO-induced suppression of DNA synthesis in lymphocytes and leukemic cells (Seyfried & Morris, 1979; Gallo et al, 1986; Endo et al, 1988). And these findings showed that DFMO produced antiproliferative effect on CEM cells without cell damages, and the antiproliferative action was exclusively dependent on the depletion of cellular polyamines and predisposed to the enhancement of apoptotic cell death induced by dexamethasone.

G_1/S transition is the critical point in cell cycle and is one of the steps to be regulated in apoptosis, and the hyperphosphorylation of pRb is appeared in G_1/S transition of cell cycle (Chiarugi et al, 1994; Kranenburg et al, 1995). ODC activity is strictly regulated during cell cycle, showing the highest peak in G_1 phase, and so polyamines have been known to be essential in cell cycle progression (Heby & Persson, 1990; Stimac & Morris, 1987; Kaminska et al, 1990). DFMO arrested the cell cycle at G_1 phase, and the G_1-arrest was definitely associated with DFMO-induced decrease of putrescine and spermidine contents in L1210 cells (Dorhout et al, 1995). Similarly in IL-3 dependent myeloid cells, the G_1-arrest induced by IL-3 deprivation also appeared to involve in the down-regulation of ODC and c-myc expression (Askew et al, 1991). In this study, the antiproliferative action of DFMO and the apoptotic action of dexamethasone described above appeared to be associated with the hypophosphorylation of pRb representing arrest of cell cycle in G_1 phase. The mechanism of hypophosphorylation of pRb induced by depletion of cellular polyamines is under study.

In conclusion, dexamethasone-induced apoptosis seems to be ascribed to cellular polyamine depletion associated with the inhibition of G_1/S transition.

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